J Infect Chemother 25 (2019) 273–280

Contents lists available at ScienceDirect

Journal of Infection and Chemotherapy

journal homepage: http://www.elsevier.com/locate/jic

Original Article

# Analysis of synergy between beta-lactams and anti-methicillin-resistant *Staphylococcus aureus* agents from the standpoint of strain characteristics and binding action

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ARTICLE INFO

*Article history:*
Received 17 December 2018
Accepted 20 December 2018
Available online 31 January 2019

*Keywords:*
MRSA
Synergistic effect
Beta-lactam
Anti-MRSA agent

ABSTRACT

In light of the increasing number of clinical cases resistant to traditional monotherapies and the lack of novel antimicrobial agents, combination therapy is an appealing solution for the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections. Here, we evaluated the efficacy of anti-MRSA agents, such as vancomycin (VAN), daptomycin (DAP), and linezolid (LZD), in conjunction with 13 beta-lactams and non-beta-lactams. We assessed the *in vitro* activities of the various combinations against 40 MRSA strains based on the maximum synergistic effect (MSE), an index calculated from the MIC change with a combination agent. Nearly all the anti-MRSA agents, which were combined with beta-lactams as well as VAN and DAP, showed a synergistic effect with arbekacin. VAN also exhibited varying degrees of synergy depending on the type of beta-lactam, whereas DAP and LZD showed similar synergy with different beta-lactams. These effects were confirmed by antibiotic kill curves, except for the apparent interaction between LZD and beta-lactams. The MSE results were analyzed according to strain characteristics including susceptibility to combination agents, staphylococcal cassette chromosome *mec* type, and presence of the *blaZ* gene; however, no obvious correlations were observed. In a fluorescence binding assay, the fluorescence intensity of boron-dipyrromethene (BODIPY)-VAN decreased, whereas that of BODIPY-DAP increased in combination with a beta-lactam agent. These findings suggest that beta-lactam combinations are promising treatment options for MRSA infections and that the type of beta-lactam combined with VAN is important for the synergistic effect.



## 1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is considered a major cause of hospital- and community-acquired infections [1,2]. Additionally, some cases are resistant to traditional monotherapies [3–5]. Thus, more effective treatment measures are needed. Vancomycin (VAN), daptomycin (DAP), and linezolid (LZD) are anti-MRSA agents that are frequently used to suppress MRSA [1]. However, in addition to facing an increasing prevalence of drug resistance, each of these agents has adverse side effects [1]. Many

*Abbreviations:* MSE, Maximum Synergistic Effect.

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https://doi.org/10.1016/j.jiac.2018.12.007
1341-321X/

novel anti-MRSA agents, such as ceftaroline, telavancin, dalbavancin, oritavancin, and tedizolid, have been developed recently and many more are under development. However, given the limited data and their side effects, none have been demonstrated as being conclusively superior to conventional anti-MRSA agents [6–8].

Beta-lactam agents—which themselves lack anti-MRSA activity owing to their poor binding affinity for penicillin-binding protein (PBP) 2a—are known to act synergistically with anti-MRSA agents *in vitro* and *in vivo*, as demonstrated in recent clinical trials [9–11]. Using existing antibiotic agents is time- and cost-saving; as such, combination therapy is an appealing solution to the shortcomings of current anti-MRSA agents.

While synergistic effects have been previously demonstrated with beta-lactam [9], different patterns of synergy have also been observed among MRSA strains; certain combinations were effective against some strains but not others [12,13]. Determining the cause of this variation could help to identify cases that are suitable for combination therapy and guide future research into the mechanisms of action of these agents. However, this has not been fully achieved to date.

The aim of the present study was to determine the optimal combinations of antibiotics that would result in high synergy as well as the relevant characteristics of the target strains. Additionally, we also investigated the mechanisms underlying the synergy between different agents. To these ends, we carried out antibiotic susceptibility testing using anti-MRSA agents and 13 combination agents, including five different beta-lactams, against 40 MRSA strains and analyzed the data about the characteristics of each strain. To elucidate the mechanisms of action, we investigated whether the binding of anti-MRSA agents varied according to the combination agent by fluorescence labeling with VAN and DAP.

## 2. Materials and methods

### 2.1. Bacterial strains

A total of 40 MRSA strains were investigated in this study; 37 were obtained from 343 MRSA isolates from different patients at the Toho University Omori Hospital in 2015 and included as many strains as possible that were resistant to each investigated antibiotic. Of the 37 strains, 13 (35%) were recovered from blood, nine (24%) from skin and soft tissue, seven (19%) from sputum, four (11%) from catheter tips (one each of central venous catheter, intubation tube, continuous ambulatory peritoneal dialysis tube, and urethral catheter), three (8%) from nose swabs, and one (3%) from vaginal secretion. Three additional control MRSA strains (BAA-1556, N315, and ATCC 43300) were also included in the analysis.

### 2.2. Antibiotic susceptibility tests

For each investigated strain, nine frozen microdilution sets of cation-adjusted Mueller Hinton broth (CAMHB) (Eiken Chemical Co., Ltd., Tokyo, Japan) were distributed in 96-well plates to evaluate the activity of the anti-MRSA agents in the presence of the combination agent. The concentrations of calcium and magnesium were 25 and 12.5 μg/ml, respectively; with DAP, the concentrations were 50 and 12.5 μg/ml, respectively. Each well of the plates contained one or two agents (anti-MRSA agent plus combination agent). The anti-MRSA agents included VAN, DAP, and LZD. The combination agents included oxacillin (OXA), cefazolin (CFZ), cefoxitin (FOX), cefmetazole (CMZ), meropenem (MEM), rifampicin (RIF), clindamycin (CLI), minomycin (MIN), trimethoprim-sulfamethoxazole (SXT), levofloxacin (LVX), clarithromycin (CLR), gentamycin (GEN), and arbekacin (ABK). A bacterial suspension containing 2–8 $\times$ $10^4$ CFU of each strain was inoculated into each well according to the guidelines of the Clinical & Laboratory Standards Institute (CLSI) M7 [14].

### 2.3. Antibiotics concentration

The optimal concentration ranges of the anti-MRSA and combination agents were determined according to the breakpoints of the CLSI and Japanese Society of Chemotherapy (JSC), and MIC distribution data from the European Committee on Antimicrobial Susceptibility Testing [15–18]. The concentrations of the individual anti-MRSA agents were determined (Table 1). Since an excessive concentration of combination agent would inhibit growth, and a too low concentration would have little effect, one to three different concentrations that included the breakpoint concentration were tested for each combination agent (Supplemental Material Table S1).

### 2.4. Combination effect

The MICs of anti-MRSA agents with and without combination agents and those of each antibiotic alone were determined according to CLSI M7 [14]. The MIC50 or MIC90—i.e., the

**Table 1**
Antimicrobial susceptibility of 40 MRSA strains to single antibiotics.

| Antimicrobial agent (measuring range, μg/ml) | MIC (μg/ml) | | Sensitive (%) |
|---|---|---|---|
| | Range | MIC 90 | |
| Oxacillin (1–4) | ≤1–>4 | >4 | 5 |
| Cefazolin (0.03–4) | 1–>4 | >4 | 15 |
| Cefoxitin (2–8) | 8–>8 | >8 | 0 |
| Cefmetazole (2–8) | ≤4–>32 | >32 | 33 |
| Meropenem (0.06–8) | 0.25–>8 | >8 | 30 |
| Rifampicin (0.002–2) | 0.008–>2 | 0.015 | 90 |
| Clindamycin (0.015–1) | ≤0.12–>1 | >1 | 25 |
| Minomycin (0.03–1) | 0.06–>8 | >8 | 60 |
| Trimethoprim-sulfamethoxazole (0.595/0.03–19/1) | ≤0.03–>1 | 0.12 | 95 |
| Levofloxacin (0.06–4) | 0.125–>4 | >4 | 18 |
| Clarithromycin (0.06–4) | 0.5–>4 | >4 | 5 |
| Gentamycin (0.03–8) | 0.25–>8 | >8 | 38 |
| Arbekacin (0.03–4) | 0.25–>4 | 2 | 95 |
| Vancomycin (0.25–2) | 0.5–1 | 1 | 100 |
| Daptomycin (0.125–1) | 0.25–>1 | 0.5 | 95 |
| Linezolid (0.5–4) | 1–4 | 2 | 100 |

concentration required to inhibit growth by 50% or 90%, respectively—was calculated for each strain. In addition, sensitive (S) rate was calculated for every single agent. Susceptibility was determined according to CLSI M100 S20/27 [15,16] except for CEZ, ABK, and SXT. When the MIC of CEZ or ABK was higher than 4 or 2 μg/ml, respectively, the strain was considered as resistant according to the JSC [17]. When the MIC of SXT was higher than 1 μg/ml, it was considered as resistant. The combination effects were assessed by the maximum synergistic effect (MSE), which was defined as the lowest value associated with one to three different concentrations for each combination and was calculated using the following formula: (MSE value) = $\log_2$ (MIC of anti-MRSA agent with combination agent)/(MIC of anti-MRSA agent alone). It was regarded as indeterminate when the MIC of the anti-MRSA agent was outside the measured range or when the single combination agent inhibited bacterial growth when applied alone. The combination effects were scored as synergistic (MSE < 0), non-existent (MSE = 0), or antagonistic (MSE > 0). Finally, the median MSE was calculated for each combination. When the value was negative, nil, or positive, the combination effect against all 40 strains was regarded as synergistic, non-existent, or antagonistic, respectively.

### 2.5. Fractional inhibitory concentration (FIC)

FIC values of one anti-MRSA agent (VAN, DAP, or LZD) plus one combination agent (CMZ, MEM, ABK, or CLI) against the control strains BAA-1556, N315, and ATCC43300 were determined as previously described [19]. Two antibiotics were selected as representative highly synergistic beta-lactam agents (CMZ and MEM), one as a highly synergistic non-beta-lactam agent (ABK), and one as a non-synergistic agent (CLI). The anti-MRSA agents were tested at seven concentrations (0.06–4 μg/ml for VAN, 0.03–2 μg/ml for DAP, and 0.25–16 μg/ml for LZD), and the combination agents were tested at 11 concentrations (0.03–32 μg/ml for CMZ, MEM, and ABK and 8–8192 μg/ml for CLI).

### 2.6. Kill curve assay

One of the following sets of antibiotics was studied against a selected MRSA strain (no. 25) grown in triplicate in CAMHB at 160 rpm and 35 °C: anti-MRSA agent (VAN, DAP, or LZD) alone, combination agent (MEM, CMZ, ABK, or CLI) alone, one MRSA agent plus one combination agent, or no antibiotic agent (growth control). Aliquots were obtained at 0, 4, and 8 h, serially diluted and plated onto nutrient agar. After overnight culture, the CFU was determined. The anti-MRSA agents were administered at the MIC of the strain, and the concentrations of combination agents were the highest as previously mentioned. The investigated strains were selected based on their MSE being calculable at the highest combination concentration and their pattern of synergistic interactions with the selected antibiotics being similar to the median of the full set of 40 strains. Only one of the 40 strains (no. 25) met these criteria.

### 2.7. Association of the combination effect with susceptibility to combination agents

The 40 MRSA strains were divided into S and intermediate/resistant (R) groups, as described above. The mean MSEs of the two groups were compared for each combination agent.

### 2.8. Association of the combination effect with staphylococcal cassette chromosome mec (SCCmec) type

Multiplex PCR 1 and 2 was performed as previously described to determine the SCC*mec* type of the investigated MRSA strains [20,21]. Briefly, after overnight culture, DNA was extracted from the cells with the phenol-chloroform method and purified. The DNA extracts were used for subsequent PCR analysis. After dividing the strains into SCC*mec* type II, type IV, and another type, the mean MSEs of type II and IV groups for each combination of agents were compared.

### 2.9. Association of the combination effect with the presence of the blaZ gene

PCR was performed as previously described to identify whether each strain harbored the *blaZ* gene [22]. The mean MSEs of strains with and without *blaZ* were compared.

### 2.10. Binding assay of VAN and DAP with the combination agent

The binding affinities of VAN for the cell wall and DAP for the cell membrane in strain 25 was determined using boron-dipyrromethene (BODIPY)-labeled VAN (BODIPY FL vancomycin; Invitrogen, Carlsbad, CA, USA) and BODIPY-labeled DAP as previously described [23,24], but with some modifications. BODIPY-DAP was synthesized according to the manufacturer's instructions [25]. The binding assay was performed as follows: the strain was grown in CAMHB to an optical density of 0.6–0.7 at 600 nm, 160 rpm, and 35 °C. Cultures were then incubated for 60 min with a combination agent (CMZ, MEPM, ABK or CLI) at the highest combination concentration for each. After washing, the cells were incubated for 20 min with 2 μg/ml BODIPY-VAN or VAN, or with 20 μg/ml BODIPY-DAP or DAP. Images were obtained at three different fields for each combination by confocal microscopy, and the mean fluorescence intensity was calculated. After subtracting the mean fluorescence intensity of VAN or DAP from that of BODIPY-VAN or -DAP, respectively, the mean fluorescence intensity of each combination was compared.

### 2.11. Statistical analysis

Data were analyzed using Prism 7 software (GraphPad Inc., San Diego, CA, USA). The coefficient of determination ($R^2$) was calculated to assess the correlation between MSE and FIC, which were regarded as correlated for values of 0.7 or higher. Differences between groups were evaluated for statistical significance with the Mann-Whitney *U* test for the sub-analysis and with the Student's t-test for the binding assay. The post-hoc test was selected based on whether the dataset was normally distributed. P values < 0.05 were considered statistically significant. For the sub-analysis, a statistical analysis was considered as not applicable (NA) when the sample size was five or less.

## 3. Results

### 3.1. MIC

All investigated strains were sensitive to VAN and LZD, and two were resistant to DAP. All strains were resistant to at least one of the two combination agents, OXA and FOX, which are used to detect MRSA. The proportion of sensitive strains varied for other antibiotic combinations (Table 1).

### 3.2. Correlation between MSE and FIC

The correlation between MSE and FIC was only observed for the VAN and MEM combination and the DAP and MEM or ABK combination (Supplemental Material Table S1). The MSE values of all anti-MRSA agents in combination with CMZ against three control strains were not determined because CMZ alone inhibited bacterial growth. On the other hand, FICs of anti-MRSA agents with CLI were not determined because the MIC of CLI alone was above the measured ranges (>8192 μg/ml). We could not determine $R^2$ for the LZD and ABK combination because the FICs for the three strains were the same.

### 3.3. Combination effect

For the combination agents for which several combinations of concentration were tested, there was generally less synergy at lower concentrations than at higher concentrations (Supplemental Material Table S2). Synergistic effects were observed when anti-MRSA agents were combined with beta-lactams (Fig. 1), but their patterns varied. VAN showed different degrees of synergy with beta-lactams; among the latter, the synergistic effect was most potent with CMZ, followed by MEM, FOX, CFZ, and OXA. In contrast, beta-lactams showed similar effects with DAP and LZD. These effects were also observed when VAN or DAP, but not LZD, was

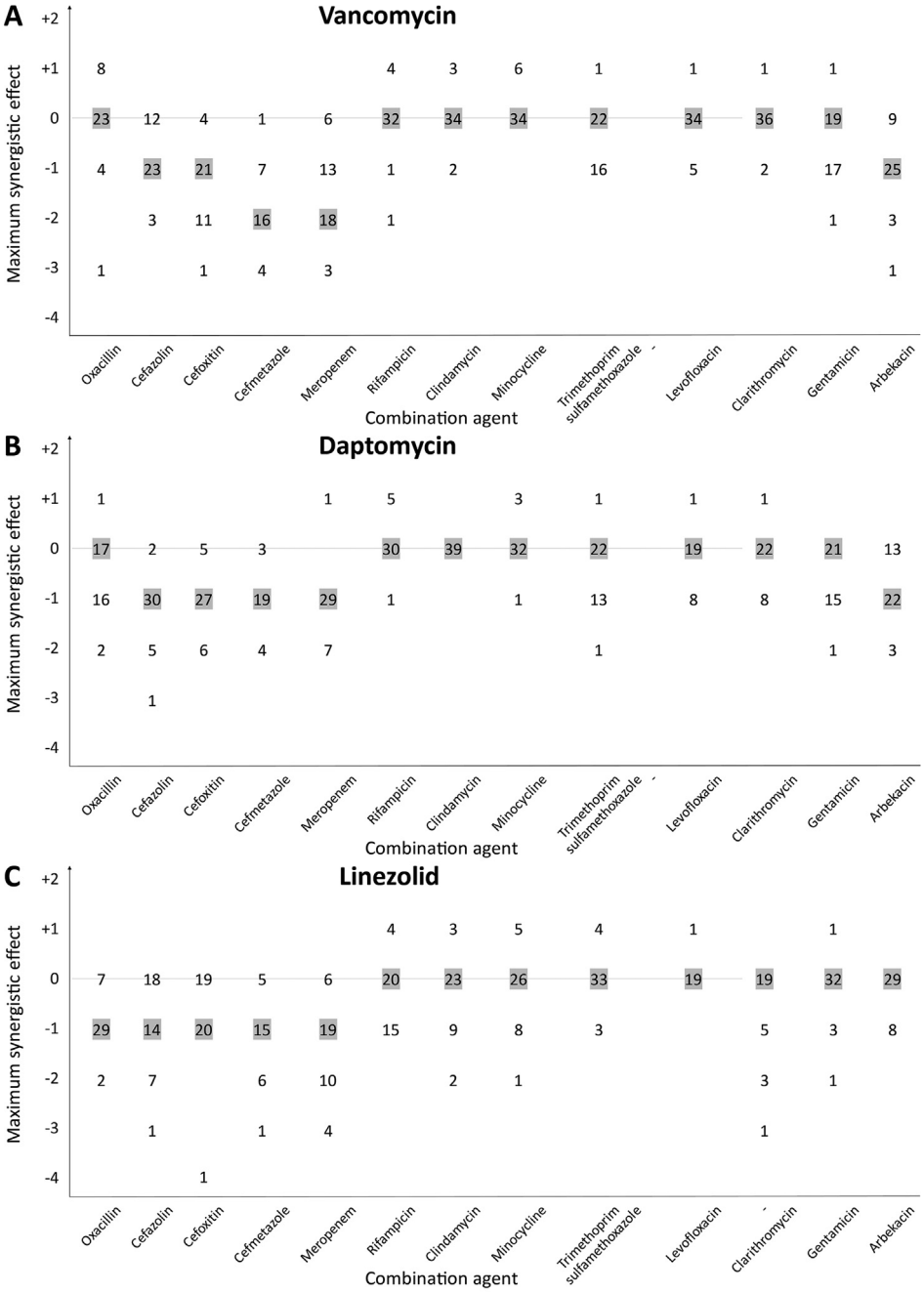


**Fig. 1.** Beta-lactams enhance the activity of anti-MRSA agents against MRSA strains. Effects of anti-MRSA antibiotics combined with other antibiotics. The vertical and horizontal axes depict MSE and each combination agent, respectively. The number of strains is shown at a point in the system of coordinates. The median MSE for each combination is shadowed. When the median MSE was negative, nil, or positive, the combination effect against all 40 strains was regarded as synergistic, non-existent, or antagonistic, respectively.

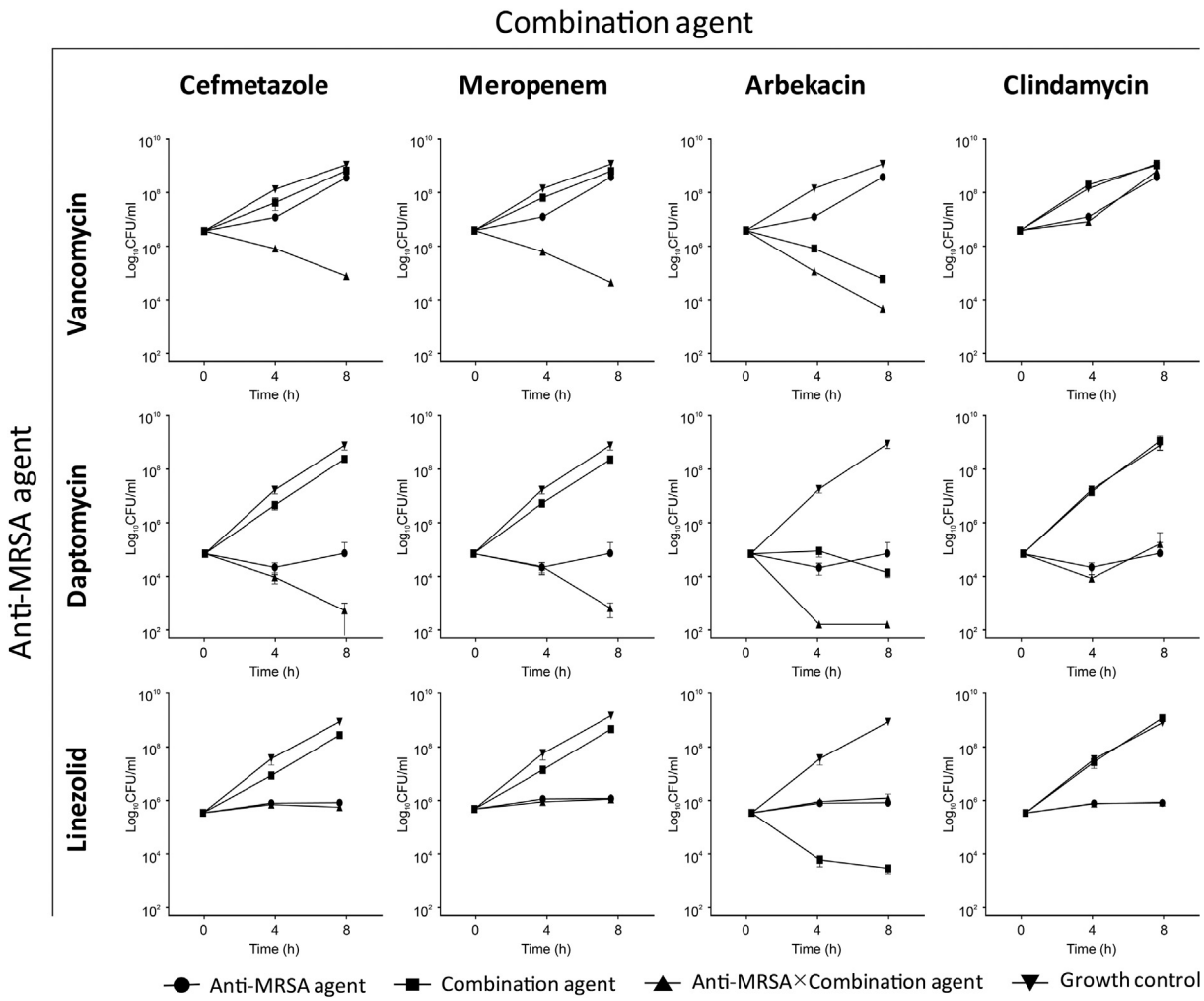


**Fig. 2.** Beta-lactams potentiate bactericidal activity of anti-MRSA agents. Time–kill curve of anti-MRSA agent (VAN, DAP, or LZD), combination agent (CMZ, MEM, ABK, or CLI) alone or in combination and growth control against strain 25. Each data point represents the mean CFU of triplicate samples, and error bars represent standard deviations. Strain 25 was considered as a representative MRSA strain. The concentrations of anti-MRSA and combination agents were the MIC of the strain and the highest concentration of the combination, respectively. In one of the triplicates for DAP with CMZ and all of the triplicates for DAP with ABK, no colonies were detected after 8 h. To calculate the average, we considered the CFU/ml of the plate without colonies as 199 CFU/ml, which was the maximum value of the CFU/ml range (0–199 CFU/ml).

combined with ABK. There were no significant synergistic effects for the other combinations.

The kill curve revealed that the combination of VAN or DAP with CMZ, MEM, or ABK was synergistic (Fig. 2). On the other hand, there was no synergy when VAN or DAP was combined with CLI or when LZD was combined with CMZ, MEM, or CLI. The combination of LZD and ABK had an antagonistic effect. In one of the triplicates of DAP with CMZ and all of the triplicates of DAP with ABK, no colonies were detected after 8 h. To calculate the average number of colonies, we considered the CFU/ml of plates without colonies as 199 CFU/ml, which was the maximum value in the tested range (0–199 CFU/ml).

### *3.4. Association of the combination effect with susceptibility to a drug combination*

No significant difference between S and R groups was observed apart from the combinations described below (results for CMZ, MEM, ABK, and CLI are shown in Table 2). The mean MSE of the R group was lower than that of the S group when LZD was combined with MEM, whereas the opposite was exact when VAN and LZD were combined with CEZ and CLI or MIN, respectively.

### *3.5. Association of the combination effect with SCCmec type*

The PCR results showed that 27, eight, and five strains harbored SCC*mec* type II, type IV, and other types (including the two non-typable strains), respectively. All strains had the *mec*A gene. No differences were observed between the type II and IV groups apart from the combinations described below (results for CMZ, MEM, ABK, and CLI are shown in Table 2). When DAP was combined with SXT and LZD with MEM, the mean MSE of the type II group was significantly lower than that of the type IV group.

### *3.6. Association of the combination effect with the presence of the blaZ gene*

The PCR analysis showed that 29 strains harbored the *blaZ* gene whereas 11 did not. The mean MSE was lower for the former group than for the latter group when VAN was combined with CMZ or MEM, DAP was combined with CMZ, and LZD was combined with OXA. There was no significant difference between groups for the other combinations (results for CMZ, MEM, ABK, and CLI are shown in Table 2).

**Table 2**
Comparison of mean maximum synergistic effect between groups in relation to MRSA strain characteristics.

| Anti-MRSA agent | Combination agent | Number of the strains | Mean maximum synergistic effect | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Susceptibility of combination agent | | | SCCmec type | | | | blaZ gene | |
| | | | R group | S group | p value | Type II | Type IV | Other type | p value | Positive | Negative | P value |
| Vancomycin | Cefmetazole | 28 | −1.7 (26) | −3.0 (2) | NA | −1.8 (23) | −1.5 (2) | −2.0 (3) | NA | −2.0 (19) | −1.4 (9) | <0.05* |
| | Meropenem | 40 | −1.6 (28) | −1.2 (12) | 0.21 | −1.5 (27) | −1.4 (8) | −1.2 (5) | 0.72 | −1.6 (29) | −1.0 (11) | <0.05* |
| | Arbekacin | 38 | 0 (2) | −0.94 (36) | NA | −0.88 (26) | −1.0 (7) | −0.8 (5) | 0.51 | −0.93 (28) | −0.8 (10) | 0.76 |
| | Clindamycin | 39 | 0.067 (30) | −0.11 (9) | 0.30 | 0 (26) | 0.13 (8) | 0 (5) | 0.34 | 0 (28) | 0.091 (11) | 0.63 |
| Daptomycin | Cefmetazole | 26 | −1.1 (25) | 0 (1) | NA | −1.0 (22) | −1.0 (1) | −1.3 (3) | NA | −1.2 (18) | −0.63 (8) | <0.05* |
| | Meropenem | 37 | −1.0 (26) | −1.1 (11) | 0.36 | −1.1 (24) | −1.1 (8) | −0.8 (5) | 0.40 | −1.2 (28) | −1.0 (9) | 0.29 |
| | Arbekacin | 38 | 0 (1) | −0.76 (37) | NA | −0.6 (25) | −1.0 (8) | −1.0 (5) | 0.16 | −0.76 (29) | −0.67 (9) | 0.84 |
| | Clindamycin | 39 | 0 (30) | 0 (9) | >0.99 | 0 (26) | 0 (8) | 0 (5) | >0.99 | 0 (28) | 0 (11) | >0.99 |
| Linezolid | Cefmetazole | 27 | −1.2 (25) | −0.50 (2) | NA | −1.1 (22) | −0.5 (2) | −1.7 (3) | NA | −1.2 (19) | −0.88 (8) | 0.39 |
| | Meropenem | 39 | −1.6 (28) | −0.64 (11) | <0.001** | −1.4 (27) | −0.75 (8) | −1.5 (4) | <0.05 | −1.4 (28) | −1.2 (11) | 0.67 |
| | Arbekacin | 37 | 0 (2) | −0.23 (35) | NA | −0.23 (26) | −0.17 (6) | −0.20 (5) | >0.99 | −0.19 (26) | −0.27 (11) | 0.91 |
| | Clindamycin | 37 | −0.067 (30) | −1.1 (7) | <0.001** | −0.12 (26) | −0.67 (6) | −0.60 (5) | 0.10 | −0.27 (26) | −0.27 (11) | 0.91 |

*P < 0.05, **P < 0.01 (Mann-Whitney *U* test). When the sample size of a group was five or less, statistical analysis was regarded as not applicable (NA). The numbers in parenthesis represent the number of the strain corresponding to the group.

### 3.7. Altered binding of VAN and DAP with combination agents

The fluorescence intensity of BODIPY-VAN was decreased in the presence of CMZ or MEM but not ABK or CLI (Fig. 3A and B). In contrast, the fluorescence intensity of BODIPY-DAP was increased with CMZ, MEM, and ABK. A small, non-significant increase was also observed with CLI (Fig. 3C and D).

## 4. Discussion

In this study, we investigated the most effective combination therapy against 40 MRSA strains based on MSE, which is more easily determined than FIC or the kill curve for a large number of strains. MSE was correlated with the value determined with the kill curve and to some extent with the FIC.

Combinations of nearly all anti-MRSA agents with beta-lactams (80% for VAN, DAP, and 100% for LZD) and of VAN or DAP with ABK had significant synergistic effects against the 40 MRSA strains. In addition, for VAN or DAP, the results of the 96-well assay for four representative agents (CMZ, MEM, ABK, and CLI) were supported by those of the kill curve assay. The observed synergistic effects of beta-lactams are consistent with those of previous reports [9]. Notably, in the present study, the pattern of synergy differed depending on the combined anti-MRSA agent type. For instance, VAN showed variable synergy depending on the beta-lactam type, whereas DAP and LZD exhibited uniform effects. For the beta-lactams, all the three anti-MRSA antibiotics showed synergistic effects in the 96-well plate assay, while only VAN and DAP, but not LZD, showed this effect in the kill curve assay. However, LZD showed synergy in the kill curve assay when the observation time was extended to 24 h (data not shown).

To identify the characteristics of high- or low-synergy stains, a large number and a variety of MRSA strains should be investigated. In this study, we examined 37 strains out of 343 clinical MRSA isolates in addition to three control strains. However, we did not find any single characteristic that could account for the differences in synergy among strains that were sub-analyzed for susceptibility to the drug combination agents, for the SCC*mec* type, or for the presence of *blaZ* gene. Therefore, these characteristics are unlikely to be the factors responsible for the synergistic effects against MRSA.

Given the negative results of the sub-analysis of strain characteristics, we assessed the differences in the extent of synergy between antibiotic agents. It was previously reported that exposure to beta-lactam combinations reduced BODIPY-VAN fluorescence intensity, but showed synergistic effects against methicillin-resistant vancomycin-intermediate *S. aureus* (VISA) and heterogeneous VISA since their cell walls were made thinner by beta-lactams [23,26]. In addition, beta-lactam exposure increased the fluorescence intensity of BODIPY-DAP, and its combinations showed synergistic effects against DAP-resistant MRSA due beta-lactam-induced change of cell membrane electric charge from positive to negative [24,26]. Given these previous findings, we examined changes in the binding of BODIPY-VAN and -DAP. We confirmed that CMZ or MEM exposure reduced the fluorescence intensity of BODIPY-VAN, whereas exposure to ABK or CLI showed no effect. Furthermore, CMZ, MEM, or ABK exposure increased the fluorescence intensity of BODIPY-DAP while only a negligible enhancement was observed with CLI. These results of the fluorescent binding assay correspond to the MSEs of the four representative antibiotic combinations, except that of the VCM and ABK combination.

In summary, we demonstrated the synergistic effects of anti-MRSA agents combined with beta-lactams, and of VAN or DAP combined with ABK using 40 MRSA strains, three anti-MRSA agents, and 13 combination agents. Combination effects were measured by MSE. The synergistic patterns of beta-lactam against

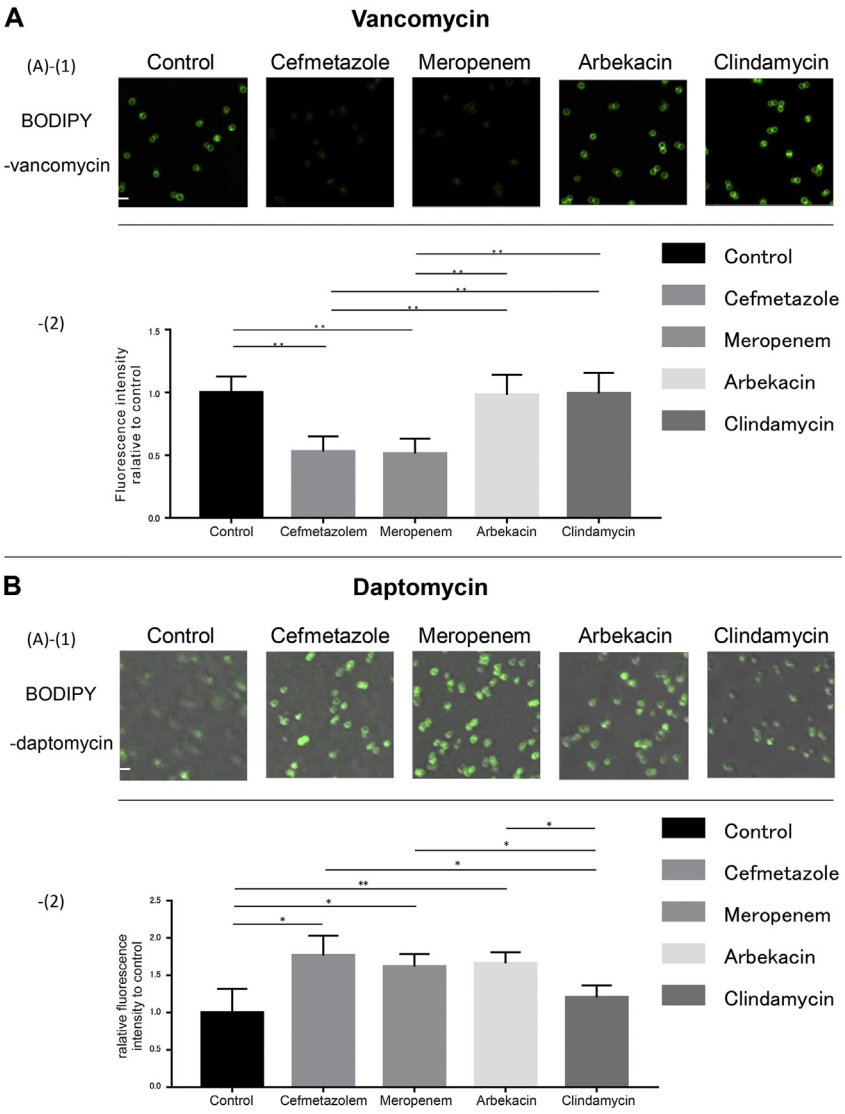


**Fig. 3.** Beta-lactams alter binding of anti-MRSA agents. Binding of fluorescent VAN and DAP decreased and increased, respectively, when cells were pre-incubated with beta-lactams. (A) Cells treated with CMZ or MEM, but not those treated with ABK or CLI, showed decreased binding to the cell wall. Scale bars, 1 μm. Images of each sample are shown at the same resolution. (B) Mean percent fluorescence intensity of BODIPY-VAN (after subtracting that of VAN) bound to strain 25 after incubation with 2 μg/ml BODIPY-VAN following pre-incubation with CMZ, MEM, ABK, or CLI. The concentrations of combination agents were 16, 4, 0.25, and 0.5 μg/ml, respectively. Error bars indicate SD. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ (Student's t-test). (C) Cells treated with CMZ, MEM, or ABK showed increased binding to the cell membrane, whereas the increase with CLI was negligible. Conditions were the same as in panel (A). (D) Mean percent fluorescence intensity of BODIPY-DAP (after subtracting that of DAP) bound to strain 25 after incubation with 20 μg/ml BODIPY-DAP following pre-incubation with CMZ, MEM, ABK, or CLI. Conditions were the same as shown in panel (B).

the 40 MRSA strains differed between VAN, DAP, and LZD. A sub-analysis of the strain characteristics including susceptibility to combination agents, SCC*mec* type, and presence of the *blaZ* gene revealed that these were not responsible for the observed synergy. Our results suggest that a change in the binding affinity of anti-MRSA agents, caused by combination agents, can explain the differences in the combination effects among the latter. These findings can provide a basis for the development of optimal drug combination strategies against MRSA.

## Funding

This work was supported by the MSD Co., Inc., U.S.A. and the Eiken Chemical Co., Ltd., Tokyo, Japan. Both the sponsors did not play any role in study design, in the collection, analysis, and interpretation of the data; nor in the writing of the report; nor in the decision to submit the article for publication.

## Conflicts of interest

Momoko Fujisaki and Hiroyuki Tojo are employees of Eiken Chemical Co., Ltd., Tokyo, Japan. All the other authors have no conflicts of interest to declare.

## Ethical approval

The study protocol was approved by the Ethics Committee of Faculty of Medicine, Toho University (No. 17-55-58).

## Acknowledgments

We thank MSD Co. Inc. U.S.A., for providing the daptomycin powder.

Editage provided English editing and Publication Support.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jiac.2018.12.007.

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